TAB F

Cannabinoid receptors as novel targets for the treatment of melanoma

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ABSTRACT   Melanoma causes the greatest number
of skin cancer-related deaths worldwide. Despite inten-
sive research, prevention and early detection are the
only effective measures against melanoma, so new
therapeutic strategies are necessary for the manage-
ment of this devastating disease. Here, we evaluated the
efficacy of cannabinoid receptor agonists, a new family
of potential antitumoral compounds, at skin melanoma.
Human melanomas and melanoma cell lines express
CB_1 and CB_2 cannabinoid receptors. Activation of
these receptors decreased growth, proliferation, angio-
genesis and metastasis, and increased apoptosis, of
melanomas in mice. Cannabinoid antimelanoma activ-
ity was independent of the immune status of the
animal, could be achieved without overt psychoactive
effects and was selective for melanoma cells vs. normal
melanocytes. Cannabinoid antiproliferative action on
melanoma cells was due, at least in part, to cell cycle
arrest at the G1-S transition via inhibition of the pro-
survival protein Akt and hypophosphorylation of the
pRb retinoblastoma protein tumor suppressor. These
findings may contribute to the design of new chemo-
therapeutic strategies for the management of melano-
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· experimental therapeutics · cell proliferation

MELANOMA CAUSES THE GREATEST number of skin
cancer-related deaths both in the USA (1) and world-
wide (2). In 2005, an estimated 59,600 Americans were
diagnosed with this cancer and 7,800 died of it (1, 3).
At present, prevention and early detection are the only
effective measures against melanoma. Thus, melanoma
basically remains a surgical disease, and excision of
thin, biologically early tumors offers the best chance of
cure. Despite intensive research, no effective therapies
exist for advanced melanoma. Only high-dose IFN α -2b

has a reproducible benefit in stage II and III patients,
but owing to its numerous side effects, modest efficacy,
and high cost, it is not used worldwide and is inconsis-
tently used in the United States (2, 4, 5). For patients
with stage IV melanoma, randomized, controlled trials
have shown no significant advantage of any specific
drug or combination of drugs. Dacarbazine, the only
cytotoxic drug approved by the FDA for the treatment
of metastatic melanoma, remains the benchmark, but it
produces responses of moderate duration and only in a
small fraction of the patients (2, 4, 5). New therapeutic
strategies are therefore necessary for the management
of melanomas.

Cannabinoid receptors, a family of G protein-cou-
pled receptors that are normally engaged by a family of
endogenous ligands—the endocannabinoids anand-
amide (6) and 2-arachidonoylglycerol (7)—and to
which active components of the hemp plant *Cannabis
sativa* L. cross-bind, participate in the control of a wide
array of biological processes (8, 9). Two different
cannabinoid receptors have been cloned from mamma-
lian tissues (8): the CB_1 receptor, mostly expressed in
brain, and the CB_2 receptor, mostly expressed in the
immune system. Preparations of cannabis have been
used in medicine for many centuries, and currently,
there is an intense renaissance in the study of the
therapeutic effects of cannabinoids focused on the
design of potent and selective synthetic cannabinoid
agonists and antagonists (8, 9). Cannabinoids have
been known to exert palliative effects in cancer patients
since the early 1970s. The best established of these
effects is the inhibition of chemotherapy-induced nau-
sea and vomiting. Today, capsules of Δ^9-tetrahydrocan-
nabinol (THC), the major active component of canna-
bis, and its synthetic analog nabilone are approved by
several countries for that purpose (10–12). Other po-
tential palliative effects of cannabinoids in oncology—
supported by phase III clinical trials—include appetite

stimulation and pain inhibition (11, 12). In addition, cannabinoids are potential antitumoral agents owing to their ability to inhibit the growth and angiogenesis of various types of tumor xenografts in animal models (11, 13–15). Cannabinoids display a fair drug safety profile in both animals and humans and do not produce the generalized cytotoxic effects of conventional chemo-therapies (11, 16–18). In this context, we have recently run a pilot clinical trial aimed at investigating the effect of THC administration on the growth of recurrent glioblastoma multiforme (19).

This background prompted us to explore the potential utility of cannabinoids as antitumoral agents against malignant melanoma. Specifically, we studied in melanoma cells *1)* the expression of cannabinoid receptors, *2)* the growth-inhibiting effect of cannabinoids, and *3)* the mechanism of cannabinoid action. Our data show that *1)* melanoma cells express functionally active CB_1 and CB_2 cannabinoid receptors, *2)* cannabinoid receptor activation inhibits melanoma cell growth *in vitro* and *in vivo*, and *3)* cannabinoids act on melanoma cells, at least in part, by arresting the cell cycle at the G1-S transition via inhibition of the prosurvival protein Akt and hypophosphorylation of the pRb retinoblastoma protein tumor suppressor.

MATERIALS AND METHODS

Cannabinoids

JWH-133 was kindly given by John W. Huffman (Department of Chemistry, Clemson University, SC), THC by Alfredo Dupetit (The Health Concept, Richelbach, Germany), and SR141716 and SR144528 by Sanofi-Aventis (Montpellier, France). WIN-55,212-2 was from Sigma (St. Louis, MO) and AM630 was from Tocris (Ellisville, MO). For *in vitro* incubations, cannabinoid agonists and antagonists were directly applied at a final DMSO concentration of 0.1–0.2% (v/v). For *in vivo* experiments, ligands were prepared at 1% (v/v) DMSO in 100 μl PBS supplemented with 5 mg/ml BSA. No significant influence of the vehicle was observed on any of the parameters determined.

Cell culture

The melanoma cell lines B16 (mouse; kindly given by José C. García-Borrón, Murcia University, Spain), A375 (human) and MelJuso (human; kindly given by Alberto Anel, Zaragoza University, Spain) were routinely maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Twenty four hours before the experiments, cells were transferred to low (0.5%)-serum DMEM.

The nontumorigenic melanocytic cell lines melan-c (mouse) and Hermes 2b (human), immortalized by telomerase reverse transcriptase expression, were kindly given by Dorothy C. Bennett (St. George's Hospital Medical School, London, UK) and Lluis Montoliu (National Biotechnology Centre, Madrid, Spain) and were maintained in 10% CO_2 atmosphere. Melan-c cells were cultured in RPMI supplemented with 10% fetal calf serum, 2 mM glutamine, 7.5 μg/ml extra phenol red and 200 nM PMA (Sigma). Hermes 2b cells were cultured in RPMI supplemented with 10% fetal calf serum, 2 mM glutamine, 7.5 μg/ml extra phenol red, 200

nM PMA, 200 pM cholera toxin (Sigma), 10 nM endothelin 1 (Sigma) and 10 ng/ml human stem cell factor (Chemicon Temecula, CA). Twenty four hours before the experiments, cells were transferred to their respective low-serum media.

Viability and cell cycle in culture

Cell viability in the cultures was determined by trypan blue exclusion. Cell cycle analysis was performed by flow cytometry determination of nuclear DNA content. Cells were detached with trypsin-EDTA (BioWhittaker, Walkersville, ME), collected by centrifugation, washed once, and incubated (1 h, room temperature) in PBS containing 1% (w/v) BSA, 30% ethanol and 5 μg/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). Fluorescence intensity was analyzed with a LSR flow cytometer (Becton Dickinson, San Jose, CA). Ten thousand cells were recorded in each determination.

Tumor induction in mice

Tumors were induced in C57BL/6 or nude mice by subcutaneous (s.c.) flank inoculation of 1×10^5 B16 melanoma cells. When tumors had reached an average volume of 300 mm³ (range, 200–400 mm³), animals were assigned randomly to the various groups and injected peritumorally (at approx. 2 mm from the tumor) for 8 days with vehicle or cannabinoid (either WIN-55,212-2 or JWH-133 at 50 μg/day, daily). Tumors were measured with external caliper, and volume was calculated as $(4\pi/3) \times (width/2)^2 \times (length/2)$.

Tumor proliferation, apoptosis and angiogenesis in mice

For proliferation assays, mice received an intraperitoneal (i.p.) injection of 5-bromo-2'-deoxyuridine (bromodeoxyuridine; 120 mg/kg body wt) (Boehringer, Mannheim, Germany) 6 h before tumor harvesting. Detection of bromodeoxyuridine (BrdU)-positive cells was performed using an anti-bromodeoxyuridine mouse monoclonal antibody (mAb) (Abcam, Cambridge, UK), as described previously (20). Apoptosis was determined with a TUNEL kit (Boehringer, Mannheim, Germany), according to manufacturer's instructions. Immunodetection of blood vessels was performed with an anti-human CD31 mAb (1:400; Cymbus Biotechnology, Hampshire, UK) as described (21). In all cases, sections were mounted with Mowiol mounting medium (Merck, Darmstadt, Germany) containing YOYO-1 iodide (1:1000; Molecular Probes, Leyden, The Netherlands) to stain cell nuclei, and fluorescence images were acquired using an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany). Morphometric analysis of the vasculature was performed with Meta-morph-Offline software, version 6.2 (Universal Imaging, Downingtown, PA). Inclusive fluorescence thresholds were set at 105 (low) and 255 (high). The number of blood vessels per area unit and the blood vessel sectional area were determined in 5–10 fields of 4–6 sections per tumor.

Generation of metastatic nodules in mice

B16 melanoma cells (5×10^5) were injected into the left paw of C57BL/6 or nude mice. Animals were injected i.p. for 21 days (C57BL/6 mice) or 12 days (nude mice) with vehicle or WIN-55,212-2 (50 μg/day, every 3 days). Lungs and livers were dissected and fixed in Bouin's solution, and the number of metastatic nodules was counted.

Cannabinoid receptor expression

Reverse transcriptase-polymerase chain reaction

RNA was isolated with the RNeasy Protect kit (Qiagen, Venlo, The Netherlands), including a DNase digestion step using the RNase-free DNase kit (Qiagen). cDNA was obtained with Transcriptor (Roche). Primers were used to amplify mRNA for human CB_1 [5'-CGTGGGCAGCCTGTTCCTCA-3' and 5'-CATGCGGGCTTGGTCTGG-3' (408-bp product)], mouse CB_1 [5'-TCTCTGGAAGGCTCACAG-3' and 5'-TGTCTGTGGACA-CAGACATG-3' (509-bp product)], human CB_2 [5'-CGCCGG-AAGCCCTCATACC-3' and 5'-CCTCATTCGGGCCATTCCTG-3 (522-bp product)], mouse CB_2 [5'-CCGGAAAAGAGGATG-GCAATGAAT-3' and CTGCTGAGCGCCCTGGAGAAC (479-bp product)] and human/mouse glyceraldehyde 3-phosphate dehydrogenase [5'-GGGAAGCTCACTGGCATGGCCTTCC-3' and 5'-CATGTGGGCCATGAGGTCCACCAC-3' (322-bp product)]. Polymerase chain reaction (PCR) reactions were performed using the following parameters: 95°C for 5 min, 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min (40 cycles), followed by a final extension step at 72°C for 5 min.

Western blot analysis

Particulate cell or tissue fractions were subjected to SDS-PAGE, and proteins were transferred from the gels onto PVDF membranes. The blots were incubated with polyclonal antibodies raised against residues 1–14 of the human/mouse CB_1 receptor (1:10000; kindly given by Allyn Howlett, NC Central University, Durham, NC) or residues 1–99 of the human CB_2 receptor (1:1000; Affinity Bioreagents, Golden, CO) as described (20). Antigen preabsorption experiments were performed by preincubating (37°C, 1 h) 1 μl (=0.5 μg) of anti-CB_1 or anti-CB_2 antibody (Ab) and 100 μl PBS with or without 20 μl (=20 μg) of the corresponding immune peptide. α-Tubulin (1:4000, Sigma) was used as a loading control. In all cases, samples were subjected to luminography with an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences, Arlington Heights, IL).

Confocal microscopy

Formalin-fixed, paraffin-embedded sections of 61 human cutaneous melanomas were obtained from the files of the Tumor Bank Network of Centro Nacional de Investigaciones Oncológicas (CNIO, Madrid, Spain). Sections (5 μm) were stained with anti-CB_1 Ab (1:500; Affinity Bioreagents, Golden, CO) or anti-CB_2 Ab (1:500; Affinity Bioreagents), as described previously (22). Slices were further incubated (1 h, room temperature, darkness) with a secondary anti-rabbit Ab-Alexa Fluor 594 (1:400; Molecular Probes, Leyden, The Netherlands). Antigen preabsorption experiments were performed by preincubating the anti-CB_1 or anti-CB_2 Ab with or without the corresponding immune peptide (Affinity Bioreagents), as described above. Control immunostainings using the secondary Ab in the absence of the primary Ab were also performed. Confocal fluorescence images were acquired using Laser Sharp 2000 software (Bio-Rad, Hercules, CA) and a Confocal Radiance 2000 coupled to Axiovert S100 TV microscope (Carl Zeiss, Oberkochen, Germany).

Expression of signaling proteins

Western blot analysis

Samples were subjected to Western blot analysis using antibodies against phospho-pRb (S807/811) (1:1000; Cell Signal-ing Technology, Beverly, MA), pRb (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), p21 (1:200; Santa Cruz Biotechnology), phospho-p27 (T157) (1:2000; R&D Systems, Minneapolis, MN), p27 (1: 1000; Santa Cruz Biotechnology), cyclin D1 (1:200; Santa Cruz Biotechnology), CDK4 (1: 1000; Pharmingen International, Bornem, Belgium), phospho-CDK2/3 (T160) (1:10000; Santa Cruz Biotechnology), phospho-p53 (S15) (1:1000; Cell Signaling Technology), p53 (1:200; Calbiochem, La Jolla, CA), phospho-Akt (S473) (1: 1000; Cell Signaling Technology), Akt (1:1000; Cell Signaling Technology), phospho-ERK (T202/Y204) (1:1000; Santa Cruz Biotechnology), ERK (1:1000; Cell Signaling Technology), phospho-p38 MAPK (T180/Y182) (1:1000; Cell Signaling Technology), p38 MAPK (1:1000; Cell Signaling Technology), phospho-c-Jun NH2-terminal kinase (T183/Y185) (1: 1000; Promega, Madison, WI) and c-Jun NH2-terminal kinase (JNK) (1:1000; Cell Signaling Technology). Densitometric analysis of the blots was performed with Multianalyst software (Bio-Rad Laboratories, Hercules, CA).

Quantitative real-time PCR

Total RNA was prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA). To assess mRNA expression, quantitative real-time PCR was performed as described (23). The generated cDNA was amplified by using primers for human p21 (5'-ATGAGTTGGGAGGAGGCA-3' and 5'-AGAAGATGTA-GAGCGGGA-3'), p27 (5'-TGGAGAAGCACTGCAGAGAC-3' and 5'-GCGTGTCCTCAGAGTTAGCC-3') and glyceraldehyde 3-phosphate dehydrogenase (5'-GGTCTTACTCCTTG-GAGGCCATGTG-3' and 5'-ACCTAACTACATGGTTTACAT-GTT-3').

Akt overexpression

Adenoviral vectors encoding enhanced GFP (EGFP) and hemagglutinin (HA)-tagged wild-type (WT) Akt (24) were amplified by infection of HEK293T cells as described (25). B16 cells were infected in serum-free medium for 1 h with 10 ml of HEK293T infected-cell supernatant, washed with PBS and transferred to 10%-serum medium for 24 h to allow expression of the adenoviral genes. Cells were seeded and 24 h before cannabinoid treatment, cells were transferred to a medium containing 0.5% serum. Infection efficiency was controlled by counting fluorescent cells on control EGFP infections and was always higher than 80%. Expression of HA-tagged Akt was confirmed in the infected melanoma cells by Western blot analysis with antibodies against total Akt (see above) and HA (1:1000; Roche, Penzberg, Germany).

Statistics

Results shown represent mean ± SD. Statistical analysis was performed by ANOVA with a post hoc analysis by the Student-Neuman-Keuls test or by unpaired Student's t test.

RESULTS

Melanoma cells express cannabinoid receptors

The expression of cannabinoid receptors in melanoma cells was examined by Western blot analysis, RT-PCR and confocal microscopy. Western blot experiments showed that CB_1 and CB_2 receptors were expressed in melanoma cell lines of murine and human origin (**Fig.**
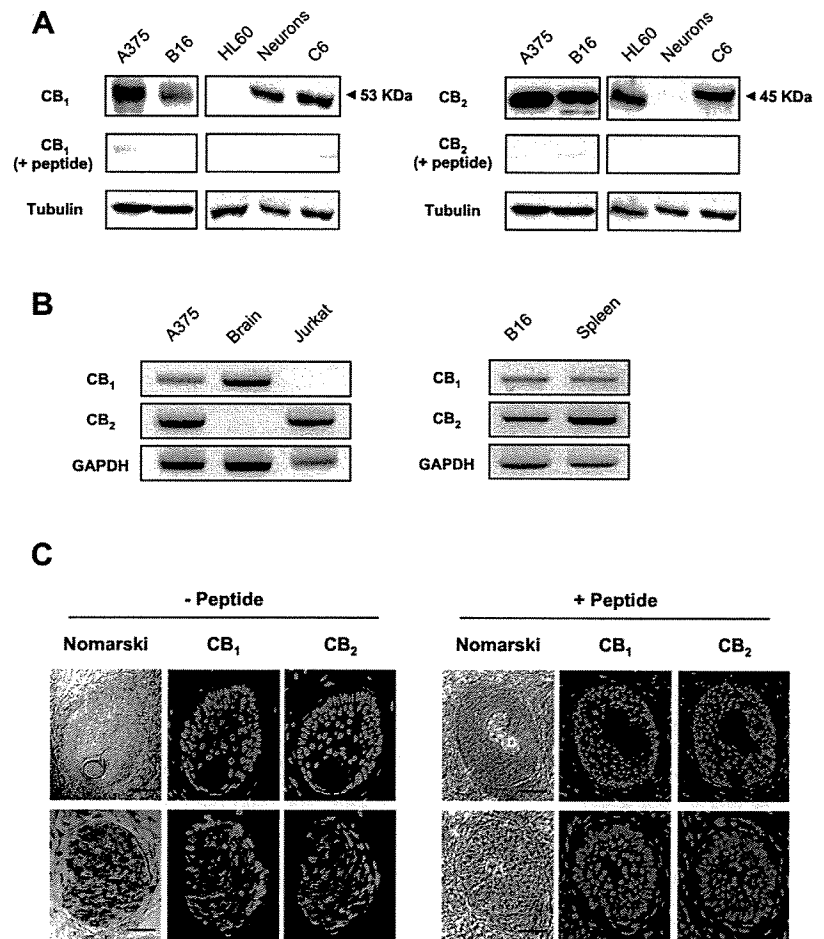
A

B

C

Figure 1. Melanoma cells express cannabinoid receptors. *A)* Western blot analysis. Human HL-60 promyelocytic cells, rat cortical neurons and rat C6 glioma cells were used as controls for CB_1 and/or CB_2 protein expression. Controls with anti-CB_1 and anti-CB_2 Ab blocking peptides are also shown. *B)* RT-PCR. Human brain-cortex extract (kindly given by María L. de Ceballos, Cajal Neuroscience Institute, Madrid, Spain), human Jurkat leukemia cells and mouse spleen were used as controls for CB_1 and/or CB_2 mRNA expression. *C)* Immunolocalization of CB_1 and CB_2 receptors (red) in two foci of a human cutaneous melanoma biopsy. Controls with anti-CB_1 and anti-CB_2 Ab-blocking peptides in two foci are also shown. Nuclei are stained in green. Scale bar: 100 μm.

1*A)*. The specificity of the anticannabinoid receptor antibodies used in the blotting experiments was assessed by antigen preabsorption with the corresponding blocking peptides. Expression of cannabinoid receptor mRNA was shown by RT-PCR (Fig. 1*B)*. Moreover, immunostaining for CB_1 and CB_2 receptors was also analyzed by confocal microscopy in a series of human cutaneous melanoma biopsies. Of the 61 tumors examined, 36 expressed significant immunoreactivity for CB_1 and CB_2 receptors, 10 just for the CB_1 receptor, 10 just for the CB_2 receptor, and only 5 were negative for the two CB receptor types. An example of CB receptor-positive tumor is shown in Fig. 1*C*. As in the blotting experiments, the specificity of the anticannabinoid receptor antibodies used for immunostaining was assessed by antigen preabsorption (Fig. 1*C)*.

Cannabinoids inhibit the growth of melanoma cells but not of normal melanocytes

We tested the functionality of cannabinoid receptors in the control of melanoma cell growth by using the plant-derived cannabinoid agonist THC and the synthetic cannabinoid agonist WIN-55,212-2. Both ligands bind to CB_1 and CB_2 receptors with comparable relative affinities and are therefore considered as mixed $CB_1/$CB_2$ agonists [reported K_i values (nM) for CB_1 and CB_2

receptors, respectively: THC = 35–80 and 4–75; WIN-55,212-2 = 2–123 and 1–16 (reviewed in Ref. 8)]. THC and WIN-55,212-2 decreased the number of viable melanoma cells in the cultures (**Fig. 2*A*, *B***). These effects were dose-dependent, and for example, values of cell density (as a percentage of the corresponding vehicle incubations) after 48 h of cannabinoid challenge were 71 ± 6 (1 μM THC), 61 ± 6 (2 μM THC), 39 ± 4 (2.5 μM THC) and 22 ± 7 (3 μM THC) for A375 cells. The growth of MelJuso cells, another human melanoma cell line, was also inhibited by cannabinoids (percentage of viability from the corresponding vehicle-treated cells after 48-h incubation: 74±3 for 100 nM WIN-55,212-2 and 70±2 for 1 μM THC; $n=4$; cannabinoids *vs.* vehicle: $P<0.01$). Cannabinoid antiproliferative action on melanoma cells relied on CB receptor activation as the CB_1 antagonist SR141716 and the CB_2 antagonists SR144528 and AM630 prevented the effect of WIN-55,212-2 in B16 and A375 cells (Fig. 2*A*, *B*). In addition, cannabinoid antiproliferative action seemed to be selective for tumor cells, as WIN-55,212-2 and THC did not induce a statistically significant change in the number of viable mouse melan-c (Fig. 2*C*) and human Hermes 2b (Fig. 2*D*) cells. These two nontumorigenic lines of melanocytes expressed CB_1 receptors (Fig. 2*C*, *D*) at levels comparable to the
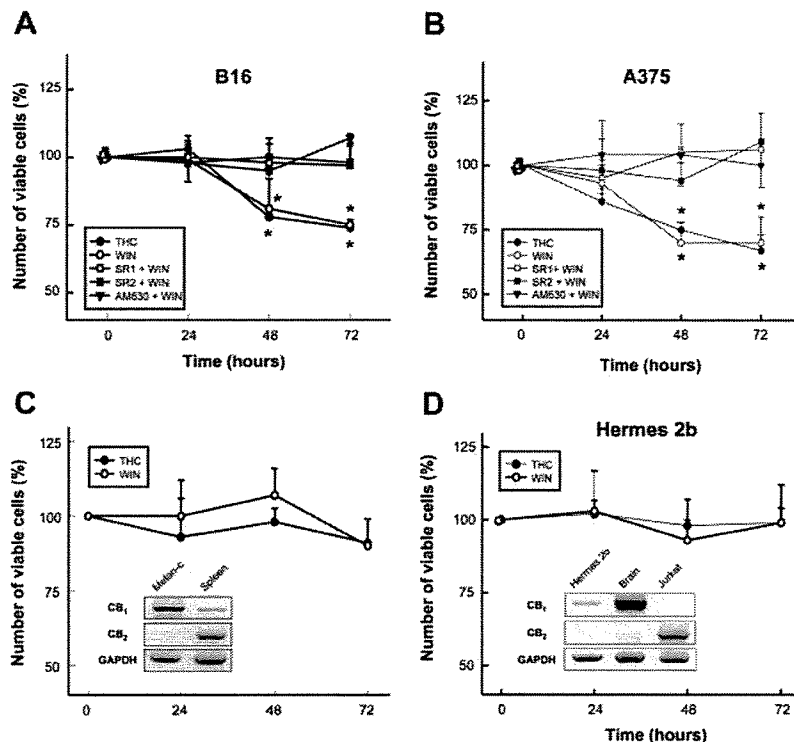
Figure 2. Cannabinoids inhibit the growth of melanoma cells but not of normal melanocytes in culture. *A, B)* The melanoma cell lines B16 *(A)* and A375 *(B)* were cultured for the times indicated with vehicle, 1 μM THC or 100 nM WIN-55,212–2, either alone or in combination with 0.5 μM SR141716 (SR1), 0.5 μM SR144528 (SR2), or 1.0 μM AM630, and the number of viable cells relative to vehicle incubations was determined ($n=5$). *Significantly different ($P<0.01$) from vehicle incubations. *C, D)* The nontransformed melanocytic lines melan-c *(C)* and Hermes 2b *(D)* were cultured for the times indicated with vehicle, 1 μM THC or 100 nM WIN-55,212–2, and cell viability was determined ($n=4$). The inset shows the RT-PCR analysis of CB_1 and CB_2 mRNA expression in the respective cell lines.

tumorigenic A375 and B16 cell lines as determined by Western blot and RT-PCR (data not shown).

Cannabinoid administration inhibits melanoma progression and metastatic spreading in mice

Given the inhibition of melanoma cell growth by cannabinoids *in vitro*, we evaluated the effect of cannabi-

noid treatment *in vivo*. Tumors generated by inoculation of the highly malignant B16 cell line were treated with vehicle or WIN-55,212–2. As shown in **Fig. 3***A*, cannabinoid administration notably reduced the growth of tumor cells *in vivo*. This antitumoral action of WIN-55,212–2 was studied in further detail.

1) Because cannabinoid-based therapeutic strategies should be as devoid as possible of psychotropic effects,
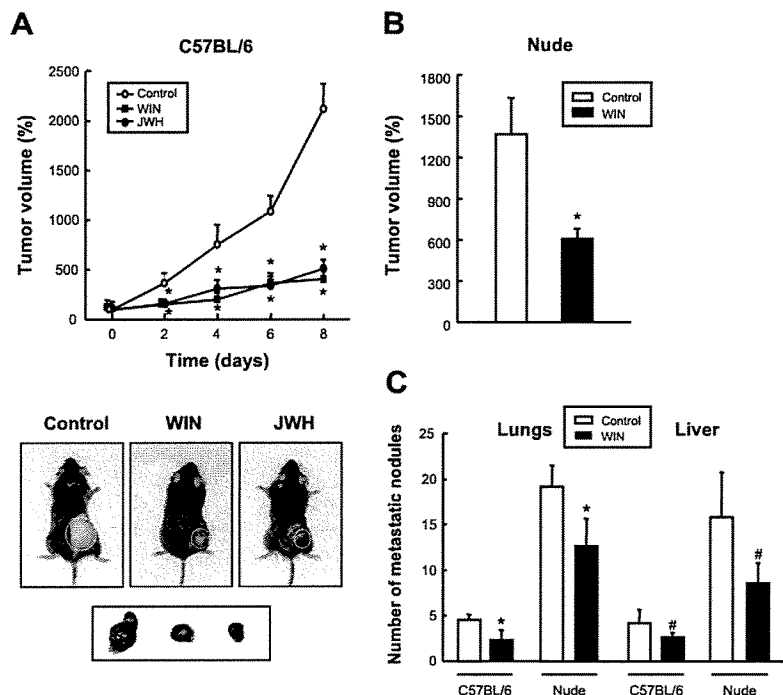

Figure 3. Cannabinoid administration inhibits melanoma progression and metastatic spreading in mice. *A)* Tumors were generated by s.c. injection of B16 cells in C57BL/6 mice, and animals were treated with either vehicle or cannabinoid (either WIN-55,212–2 or JWH-133 at 50 μg/day, daily) for up to 8 days ($n=8$ for each experimental group). Tumor size was monitored during the treatment. Examples of tumors in the flank of mice and after dissection are shown. *Significantly different ($P<0.01$) from vehicle administration. *B)* Tumors were generated by s.c. injection of B16 cells in nude mice, and animals were treated with either vehicle or WIN-55,212–2 (50 μg/day, daily) for 8 days ($n=6$ for each experimental group). *Significantly different ($P<0.01$) from vehicle administration. *C)* B16 cells were injected intraplantarly in C57BL/6 and nude mice, and animals were treated with either vehicle or WIN-55,212–2 (50 μg/day, every 3 days) for 21 days (C57BL/6 mice, $n=6$) or 12 days (nude mice, $n=5$). The number of metastatic nodules in the lungs and the liver were subsequently counted. Significantly different ($\#P<0.05$, $*P<0.01$) from vehicle administration.

which are mediated by brain CB_1 receptors (9), and melanoma cells express CB_2 receptors (see above), which lack cannabinoid psychoactivity, we administered to mice JWH-133, a nonpsychoactive, CB_2-selective agonist. As shown in Fig. 3A, JWH-133 was as effective as the mixed CB_1/CB_2 agonist WIN-55,212-2 in preventing tumor growth.

2) To distinguish between direct WIN-55,212-2 antitumoral action on melanoma cells and potential immune-related responses induced by cannabinoid treatment, parallel experiments were conducted in immune-deficient (nude) mice. As shown in Fig. 3B, WIN-55,212-2 significantly inhibited melanoma growth in these animals.

3) To test whether the antitumoral effect of WIN-55,212-2 also targets melanoma cell spreading, we examined cannabinoid action in a model of metastatic-nodule formation. As shown in Fig. 3C, WIN-55,212-2 decreased the number of lung and liver metastases after systemic inoculation of melanoma cells to C57BL/6 and nude mice.

We next tested whether, as occurs in cultured melanoma cells, cannabinoids inhibit proliferation of tumor cells *in vivo*. Quantification of proliferative (=bromo-deoxyuridine (BrdU)-labeled) cells in tumor sections revealed that treatment with WIN-55,212-2 or JWH-133

decreased tumor cell proliferation (**Fig. 4A**). This was accompanied by an increase in the number of apoptotic cells, as determined by TUNEL staining (Fig. 4B), and by a decrease in tumor vascularization, as determined by CD31 immunostaining (Fig. 4C). Morphometric analysis of the vasculature showed that the latter effect was associated with a decrease in vascular density (number of blood vessels per area unit: vehicle, 1.16 ± 0.10; WIN-55,212-2, 0.44 ± 0.08; JWH-133, 0.48 ± 0.07; $n=4$; cannabinoids *vs.* vehicle: $P<0.01$) and not to changes in vessel size (relative blood vessel sectional area: vehicle, 100 ± 16; WIN-55,212-2, 112 ± 36; JWH-133, 110 ± 35; $n=4$).

Cannabinoids inhibit melanoma cell cycle

The mechanism by which cannabinoids inhibit melanoma cell proliferation was investigated. Flow cytometry analysis showed that cannabinoid treatment inhibited B16 and A375 melanoma cell cycle, most likely at the G1-S transition, as shown by the increase of cells in the G0/G1 compartment and the decrease of cells in the S compartment (**Fig. 5A, B**). At the doses used in these experiments, no apoptotic effect of WIN-55,212-2 and THC was evident as inferred from the
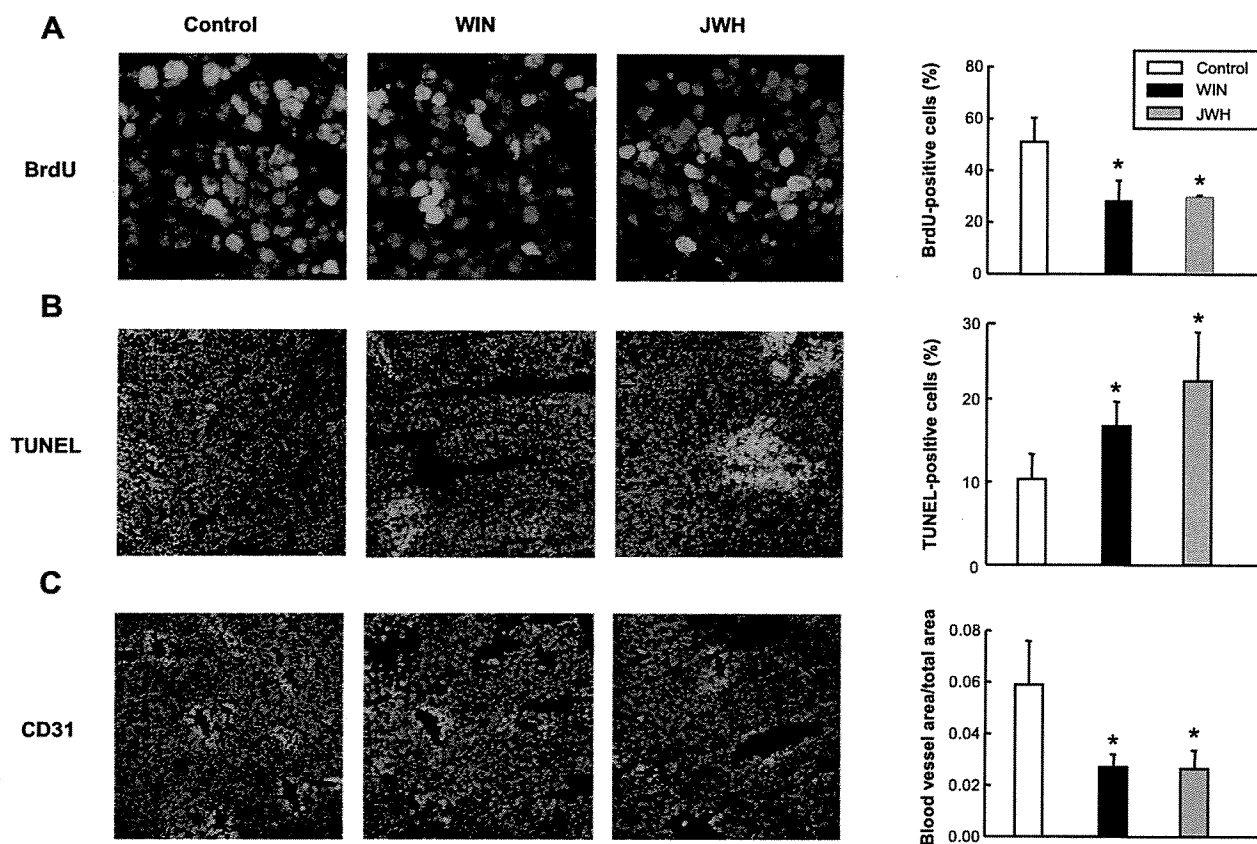

Figure 4. Effect of cannabinoid administration on melanoma cell proliferation, apoptosis, and angiogenesis in mice. Tumors were generated by s.c. injection of B16 cells in C57BL/6 mice, and animals were treated with either vehicle, WIN-55,212-2, or JWH-133 for 8 days. Five to ten fields of four to six sections from four different tumors were analyzed per group. Representative images are shown in each case. A) BrdU incorporation. B) TUNEL staining. C) CD31 immunostaining. *Significantly different ($P<0.01$) from vehicle administration.
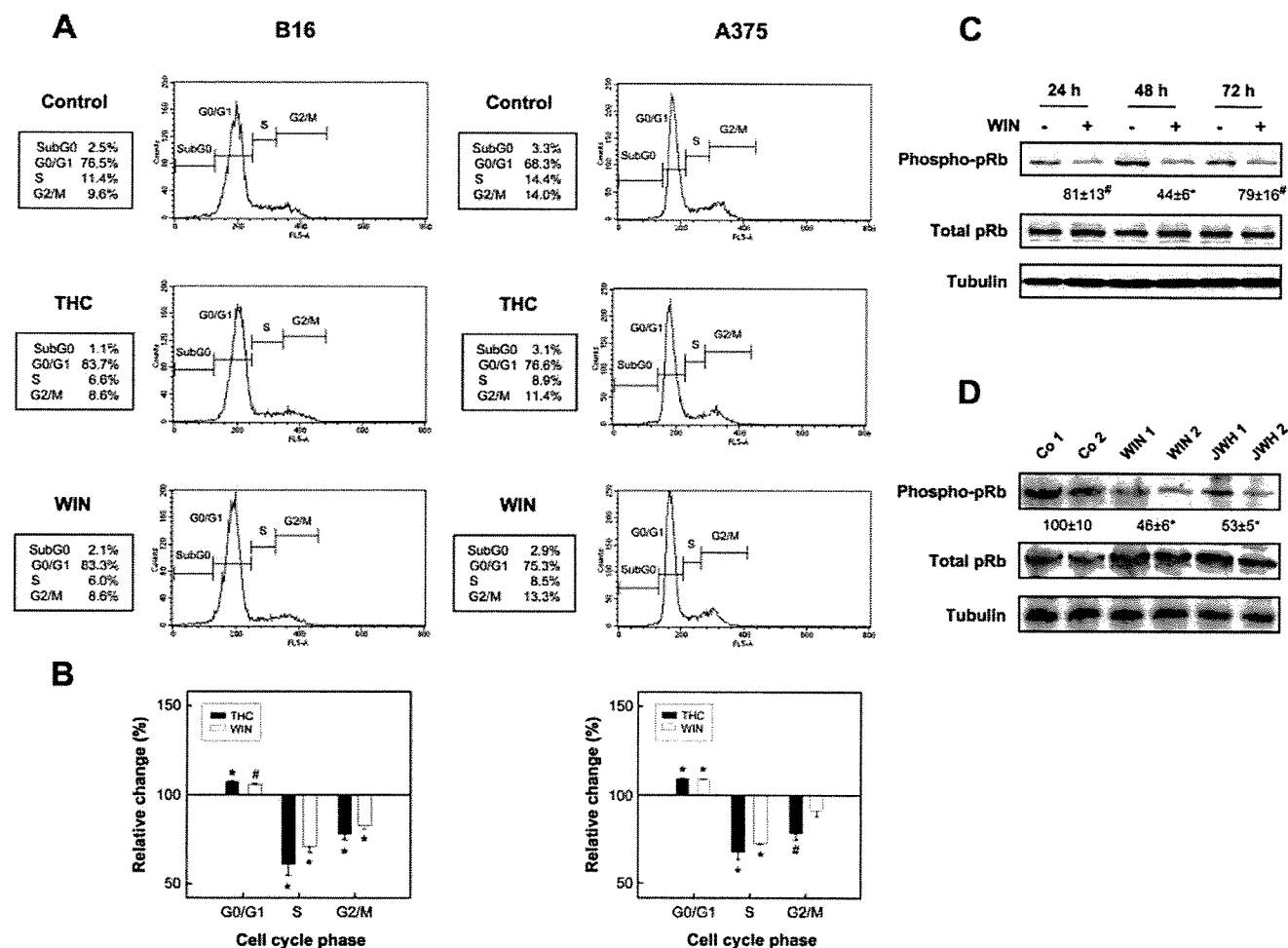
Figure 5. Cannabinoids inhibit melanoma cell cycle. *A, B)* Cells were cultured for 48 h with vehicle, 100 nM WIN-55,212-2, or 1 μM THC, loaded with Hoechst 33342, and the cell cycle was analyzed by flow cytometry. Examples of cell cycle profiles are shown in (*A*). The numbers indicate the percentage of cells at the different compartments. Quantification of the cannabinoid effect in 6 different experiments is shown in (*B*). Significantly different ($^{\#}P<0.05$, $^{*}P<0.01$) from vehicle incubations. *C)* B16 cells were cultured for the times indicated with vehicle or 100 nM WIN-55,212-2, extracts were obtained and Western blot analysis was performed. One representative experiment is shown. Optical density (OD) values (in percentage of the respective vehicle incubations; $n=4$) are given for phospho-pRb relative to total pRb. Significantly different ($^{\#}P<0.05$, $^{*}P<0.01$) from vehicle incubations. *D)* Extracts were obtained from the tumors described in Fig. 4, and Western blot analysis was performed. Two representative tumors are shown for each condition. OD values ($n=4$) are given as in (*C*). *Significantly different ($P<0.01$) from vehicle administration.

analysis of the sub-G0/G1 compartment. However, at higher concentrations ($\geq$2 μM for THC, $\geq$0.5 μM for WIN-55–212-2), both cannabinoids were also able to induce apoptosis as determined by *1)* appearance of a hypodiploid (sub-G0/G1) population, *2)* TUNEL staining, and *3)* caspase 3 activation (data not shown).

We next analyzed the levels of various proteins that are involved in the control of the cell cycle at the G1-S transition. In line with the aforementioned flow cytometry data, cannabinoids decreased the phosphorylation state of the retinoblastoma protein family member pRb, a master regulator of the G1-S transition (26), both in cultured melanoma cells (**Fig. 5C**) and in tumor xenografts (Fig. 5D). In contrast, no significant effect of cannabinoid challenge was observed on the levels of other classical members of the G1-S transition machinery, such as the cyclin-dependent kinases CDK2/3 and

CDK4; the CDK inhibitors p21 (WAF1; both protein and mRNA), and p27 (KIP1; both phosphorylated and total protein as well as mRNA); and the CDK4/6 activator cyclin D1 (Supplemental Fig. 1). In addition, the levels of total p53 and phospho-Ser-15-p53 were unaffected by cannabinoid treatment (Supplemental Fig. 1).

Akt is involved in cannabinoid-induced inhibition of melanoma cell proliferation

Cannabinoid receptors are known to modulate several pathways that are directly involved in the control of cell proliferation and survival, including phosphatidylinositol 3-kinase/Akt (27) and the extracellular signal-regulated kinase (ERK) (28), c-Jun N-terminal kinase (c-Jun

Figure 6. Akt is involved in cannabinoid-induced inhibition of melanoma cell proliferation. *A)* B16 cells were cultured for the times indicated with vehicle, 100 nM WIN-55,212–2, 1 μM THC, or 100 nM JWH-133; extracts were obtained, and Western blot analysis was performed. One representative experiment is shown. OD values (in percentage of the respective vehicle incubations; $n=4$) are given for phospho-Akt relative to total Akt in short-term incubations. *Significantly different ($P<0.01$) from vehicle incubations. *B, C)* B16 cells were infected with HA-Akt or empty vector and further cultured for 48 h with vehicle or 100 nM WIN-55,212–2. The number of viable cells (*B*) and pRb phosphorylation status (*C*) were determined. Western blot controls of Akt expression in the different infection conditions and OD values for pRb are shown. Results correspond to four different experiments. *Significantly different ($P<0.01$) from vehicle incubations.
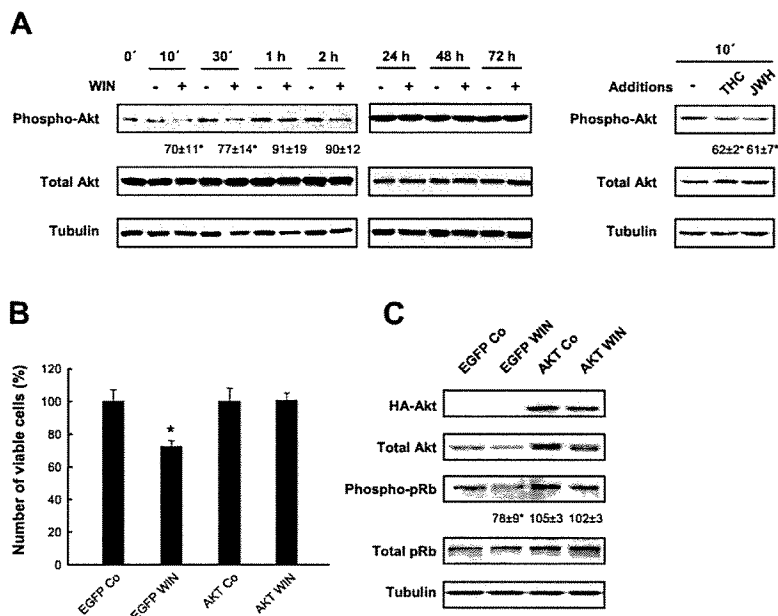
NH2-terminal kinase) (29, 30), and p38 mitogen-activated protein kinase (MAPK) (29, 30) cascades. We, therefore, examined the possible contribution of these pathways to the inhibition of B16 melanoma cell proliferation. Cannabinoid incubation induced a rapid inhibition of the prosurvival protein Akt (**Fig. 6A**), whereas ERK, JNK and p38 MAPK were not significantly affected (Supplemental Fig. 2).

To test the involvement of Akt in the growth-inhibiting effect of cannabinoids, we overexpressed Akt in B16 melanoma cells. We used an adenoviral vector to ensure that nearly all of the cells expressed the exogenous protein. Akt overexpression abrogated the antiproliferative effect of WIN-55,212–2 (Fig. 6*B*), as well as cannabinoid-induced pRb hypophosphorylation (Fig. 6*C*), suggesting that Akt inhibition is necessary for cannabinoid action.

DISCUSSION

Melanoma remains a management challenge. Despite many years of intensive research, currently approved therapies—high-dose IFN α-2b and dacarbazine—are only palliative or even ineffective, and ongoing therapeutic approaches, such as vaccine-based immunotherapy and targeted chemotherapy are as yet far from the clinics (2, 4, 5). Here, we studied the potential efficacy of cannabinoids as antitumoral agents against melanoma and show that these compounds exert a remarkable growth-inhibiting effect on melanoma cells *in vivo* that is evident under various experimental settings (animals with different immune statuses, melanoma cells inoculated at different sites, cannabinoids injected by different routes). Moreover, this is associated with an improvement of various tumor-progression parameters (decreased proliferation and vascularization, increased

apoptosis), as well as with an inhibition of tumor-cell metastatic spreading, one of the clinical hallmarks of advanced melanoma. In addition, cannabinoid action—at least *in vitro*—was selective for tumor *vs.* nontumor cells, in line with previous studies on cells of astroglial (25, 31), epidermal (20), and thyroid-epithelial (32) origin, in which cannabinoids were able to kill tumor cells but not their nontransformed counterparts. Likewise, in primary astrocytes (25), oligodendrocytes (33) and mast cells (34), as well as in Chinese hamster ovary cells transfected with the CB_1 receptor cDNA (27), cannabinoids activate Akt, while in glioma cells they inhibit Akt (35). These and other findings support that cannabinoid receptors regulate cell survival pathways differently in tumor and nontumor cells and support the idea that cannabinoids are not expected to produce the generalized cytotoxic effects of conventional chemotherapies (11, 12).

Activation of cannabinoid receptors decreases melanoma cell proliferation, at least in part, via inhibition of Akt, a key element of a major prosurvival pathway that is deregulated in many types of tumors, including melanoma (5, 36, 37). Thus, Akt is constitutively activated in more than 60% of melanomas, with higher frequency of activation at later stages of the disease (2, 5). Several mechanisms may account for Akt overactivation in melanomas. For example, melanocytic lesions frequently show loss of PTEN, a lipid phosphatase that prevents Akt activation by phosphatidylinositol 3-kinase and which heterologous expression inhibits melanoma cell growth in culture and in nude mice (38). Loss of heterozygosity on regions of chromosome 10q, which harbors the PTEN locus, has been demonstrated in 30–50% of malignant melanomas, and ~10% of melanomas carry PTEN mutations (2, 38). Akt overactivation in melanomas may also be due to amplification of the AKT3 gene (39) and to mutations of the RAS gene

family, particularly NRAS, which appear in 10–20% of melanomas (40). Our findings also support that Akt inhibition arrests the cell cycle at the G1-S transition via pRb retinoblastoma protein hypophosphorylation. However, we cannot rule out that cannabinoid antiproliferative action in our system also relies on additional mechanisms.

The observed modulation of Akt but not of MAPK cascades in melanoma cells supports the pleiotropic coupling of cannabinoid receptors to different signaling pathways in different cell contexts. For example, it has been reported that cannabinoid receptors activate ERK (41) and Akt (25) in primary astrocytes, activate ERK (42) but do not affect Akt (27) in promyelocytic HL-60 cells, and activate ERK (14) and inhibit Akt (35) in C6 glioma cells. The precise reasons for these various signaling outcomes are as yet unclear, but as discussed elsewhere (11), it is conceivable that experimental factors such as the nature of the (endo)cannabinoid ligand (*e.g.*, stability and hydrophobicity), the strength of signal input (*e.g.*, agonist potency, dose and time of exposure), the biological properties of the target cell (*e.g.*, origin, stage of differentiation and replicative capacity) and its cannabinoid receptors (*e.g.*, number and G protein coupling), and the presence of various factors in the culture (*e.g.*, growth factors and other cell types/clones) may affect cannabinoid receptor signaling properties.

Although the use of cannabinoids in medicine is limited by their psychotropic effects, these compounds display a fair drug safety profile; their potential adverse effects are within the range of those accepted for other medications—especially in cancer treatment—and their psychoactive effects tend to disappear with tolerance on continuous use (11, 12). Nonetheless, it is obvious that cannabinoid-based therapies devoid of side effects would be desirable. As the unwanted effects of cannabinoids are mediated largely or entirely by CB_1 receptors within the brain, the most conceivable possibility would be to use cannabinoids that selectively target CB_2 receptors. Although under certain circumstances CB_2 receptor activation in immune cells may blunt host antitumor activity, as discussed elsewhere (11, 43), this may be evident only in particular experimental conditions, and indeed most of the data obtained so far from animal models of cancer have demonstrated a tumor growth-inhibiting action of cannabinoids (11). In this context, here, we show that CB_2 receptor activation is functional in curbing melanoma cell growth *in vitro* and *in vivo*, the latter under conditions previously observed to produce no overt signs of psychoactivity (43). The apparent synergy between CB_1 and CB_2 receptors in inhibiting melanoma cell growth, as suggested by pharmacological antagonism experiments, is nonetheless intriguing, as it is not observed in the case of cannabinoid-induced apoptosis of glioma cells (14, 43). Of interest, nontransformed melanocytes only expressed CB_1 receptors, while melanoma cells expressed CB_1 and CB_2 receptors (*cf.* 44), in line with our previous observation the CB_2 receptor is expressed in high-grade gliomas but not in primary astrocytes (43). Hence, the present report, together with the implication of CB_2 receptors in the control of processes such as pain initiation (45), emesis (46), and inflammation (47), opens the attractive possibility of finding cannabinoid-based therapeutic strategies devoid of nondesired psychotropic side effects. Specifically, the antiproliferative effect of cannabinoids reported here may set the basis for a new therapeutic approach for the treatment of malignant melanoma. **FJ**

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